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4	Claims	4	INF C12N15/42
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6	C.	6	INF. G01N 33/564.

AQ2

(57) A Mycobacterium bovis BCG polypeptide having a molecular mass of about 64 kD was found to be useful as an immunogen inducing resistance to autoimmune arthritis and similar autoimmune diseases.

The invention relates to the use of this polypeptide for the preparation of compositions for the alleviation, treatment and diagnosis of autoimmune diseases, especially arthritis conditions.

The invention also relates to a polypeptide comprising the epitope essential for this activity. The polypeptide has the formula

171 GLITVEESNT 181 FGLQLELTEG 191 MRFDKGYISG 201 YFVTDPERQE
211 AVLEDPYILL 221 VSSKLSTVKD 231 LLPLLEKVIG.

Further, the invention relates to polypeptide showing sequential homology with said polypeptide, and to derivatives and multimers thereof. Also, microorganisms expressing the polypeptides either as such or as part of a fusion protein or as a multimer, form part of the invention.

Finally, the invention relates to pharmaceutical compositions, diagnostic compositions and test kits comprising a compound according to the invention.

rabbit sera. The article states that among 770 clones tested, several were found that produced various mycobacterial antigens in low amounts, with concentrations generally close to the detection limit. One particular clone was chosen for further investigation. This clone produced a 64 kD antigen. By placing the lambda promoter P_L in front of the structural gene of this antigen, an overproducing E. coli strain was obtained. The article shows that antigens cross-reacting with the 64 kD protein are present in a wide variety of mycobacteria and also in so-called purified protein derivatives which are routinely used for skin tests. Finally, it is stated in the article that preliminary experiments indicate the presence of antibodies against the 64 kD antigen in sera from tuberculosis patients.

According to the present invention, Antigen A was found to have the following amino acid sequence:

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   1 fMAKTIAYDE ARRGLERGLN ALADAVKVTL GPKGRNVVLE KKWGAPTITN DGVSIAKEIE
  61  LEDPYEKIGA ELVKEVAKKT DDVAGDGTTT ATVLAQALVR EGLRNVAAGA NPLGLKRGIE
 121  KAVEKVTETL LKGAKEVETK EQIAATAAIS AGDQSIGDLI AEAMDKVGNE GVITVEESNT
 181  FGLQLELTEG MRFDKGYISG YFVTDPERQE AVLEDPYILL VSSKVSTVKD LLPLLEKVIG
 241  AGKPLLIIAE DVEGEALSTL VVNKIRGTFK SVAVKAPGFG DRRKAMLQDM AILTGGQVIS
 301  EEVGLTLENA DLSLLGKARK VVVTKDETTI VEGAGDTDAI AGRVAQIRQE IENSDSDYDR
 361  EKLQERLAKL AGGVAVIKAG AATEVELKER KHRIEDAVRN AKAAVEEGIV AGGGVTLLQA
 421  APTLDELKLE GDEATGANIV KVALEAPLKQ IAFNSGLEPG VVAEKVRNLP AGHGLNAQTG
 481  VYEDLLAAGV ADPVKVTRSA LQNAASIAGL FLTTEAVVAD KPEKEKASVP GGGDMGGMDF
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Detailed discussion of the invention

As mentioned above clones A2b and A2c as disclosed in EP A O 181 364 can be used to identify antigens associated with arthritogenicity or with suppression of arthritogenicity. Both clones respond to whole mycobacterial and both A2b and A2c respond to antigen A.

T-cell clones A2b, and A2c and control cell-line Cla (anti-ovalbumin) were assayed for in vitro proliferative responses to Micobacterium tuberculosis, Antigen A, E. coli control lysate, ovalbumin (OVA) and mitogen ConA in a standard test (20 x 10^3 clone line cells, 2 x 10^5 irradiated accessory cells and antigens in optimum concentration per well, ^{3}H-Thymidine incorporation for 18 hours after 48 hours of incubation). The following table A shows the test results which are expressed as stimulation indexes.

TABLE A.

	M. tub.	Ant. A	coli contr.	OVA	ConA
A2b	180	500	2.9	-	430
A2c	304	516	1.5	-	390
Cla	-	1.5	1.2	45	64

The in vivo potency of Antigen A was checked by immunizing rats with Antigen A before and after induction of arthritis with M. tuberculosis. The test with challenge after immunization was carried out as follows:

Groups of 4 Lewis rats wer treated by intraperitoneal inoculation of water, Antigen A (50 µg) and E. coli control lysate (amount equivalent to coli content of 50 µg Antigen A) in oil. 35 Days later, susceptibility to induction of adjuvant arthritis was tested by inoculating the rats intracutaneously with M. tuberculosis (1 mg) in oil. Occurrence of arthritis was checked by daily inspection of the rat joints. The results are shown in table B.

TABLE I.

Cross-reactivity between Antigen A and antigens present in other bacteria.

	Antig.A	64kD of mycobact.	E.coli 60kD	Treponem.	Shig.	Salmon.	Klebsiella
MCA HATR							
1-24	+	-	+	+	+	+	+
F47-1C	+	+	+	+	+	+	+
Polycl.anti com.ag. Legion./ Pseudom.	+	+	+	+	+	+	+

Serological cross-reactivity as shown by Western-blot analysis. HATR 1-24 and F47-1C are monoclonal antibodies raised against Treponema and Mycobacterium tuberculosis respectively.

The polyclonal serum was raised against the common antigen of Legionella and Pseudomonas.

This indicates that epitopes present on Antigen A are similarly present on presumably equivalent proteins of various bacterium species, such as from Mycobacterium, Escherichia, Treponema, Shigella, Salmonella, Yersinia, Nocardia, Campylobacter, or Klebsiella species. Particularly, antigen A amino acid sequence 190-213 is also present in a corresponding 65 KD protein from Mycobacterium lepraea, with the exception that, in the M. leprae protein, amino acid 206 is not proline, but alanine.

Further, it was found that only part of the Antigen A sequence is responsible for the stimulating activity upon T-cell clones A2b and A2c. This was determined by testing Antigen A fragments, namely truncated derivatives produced by deletion mutants of the gene, fusion proteins with β-galactosidase and proteolysis products of Antigen A, for their ability to stimulate said T-cell clones. These fragments were obtained by means of recombinant-DNA techniques, by incorporating parts of the Antigen A gene, in some cases fused to the β-galactosidase gene, into a plasmide and expressing in E. coli K12 M1070.

The peptide with Antigen A amino acid sequence 234-540 was shown not to stimulate clones A2b and A2c. However, the fragment lacking amino acid sequence 481-540 did. β-Galactosidase-fused peptides with Antigen A amino acid sequence 61-540, 109-540 and 171-540 were reactive, those with amino acid sequences 272-540 and 280-540 were not reactive. β-Galactosidase alone was not reactive.

Therefore, the epitope responsible for the stimulation of T-cell clones A2b and A2c resides in amino acid sequence 171-234.

In order to further characterize the area which is essential for the T-cell epitopes, protease digests of Antigen A were tested for their stimulating activity on both T-cell clones. Digesting Antigen A with clostripain yielded only one reactive mixture of two peptides. The mixture is called CP15. The two peptides, which were not separated, are designated as CP15a and CP 15b. The CP15a sequence begins with amino acid 193 and that of CP15b starts with amino acid 197.

Digesting CP15 with trypsin, again, yielded a reactive mixture of two peptides (CP-TP-T12a and b) with sequences beginning with amino acid 193, and 196, respectively, as well as a non-reactive peptide, the sequence of which starts with amino-acid 209. The carboxy ends of the peptides were not determined.

It may be concluded from these results that the epitope responsible for the stimulation of T-cell clones A2b and A2c resides in Antigen A amino acid sequence 193-234, and more specifically in the amino acid sequence 193-208.

means so as to establish the presence and degree of lymphocyte activation: amongst these there may be mentioned:

a. production of lymphokines (such as interleukin-2-(IL-2));

b. gamma interferon;

c. migration inhibition factor (MIF);

d. expression of membrane markers, such as IL-2 receptor; peanut agglutination receptor;

e. expression of enzymes such as heparanase.

b. determination of antibody titer in absolute terms or as a ratio of the values obtained by different compositions, said values or ratios being indicative of the presence or absence of the disease. Quantitative values obtained are of use in establishing the severity of the disease.

The diagnostic compositions according to the invention may be prepared by combining one or more antigenic compounds according to the invention as above-defined with suitable adjuvants and auxiliary components. Standardized kits with reference and calibration means are of value in the rapid and convenient determination of arthritic disease and its stage and/or severity.

Claims

1. Use of peptide of the formula

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  1 MAKTIAYDEE ARRGLERGLN ALADAVKVTL GPKGRNVVLE KNWGAPTITN DGVSIAKEIE
 61  LEDPYEKIGA ELVKEVAKKT DDVAGDGTTT ATVLAQALVR EGLRNVAAGA NPLGLKRGIE
121  KAVEKVTETL LKGAKEVETK EQIAATAAIS AGDQSIGDLI AEAMDKVGNE GVITVEESNT
181  FGLQLELTEG MRFDKGYISG YFVTDPERQE AVLEDPYILL VSSKVSTVKD LLPLLEKVIG
241  AGKPLLIIAE DVEGEALSTL VVNKIRGTFK SVAVKAPGFG DRRKAMLQDM AILTGGQVIS
301  EEVGLTLENA DLSLLGKARK VVVTKDETTI VEGAGDTDAI AGRVAQIRQE IENSDSDYDR
361  EKLQERLAKL AGGVAVIKAG AATEVELKER KHRIEDAVRN AKAAVEEGIV AGGGVTLLQA
421  APTLDELKLE GDEATGANIV KVALEAPLKQ IAFNSGLEPG VVAEKVRNLP AGHGLNAQTG
481  VYEDLLAAGV ADPVKVTRSA LQNAASIAGL FLTTEAVVAD KPEEEKASVP GGGDMGGMDF
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for the preparation of compositions for the alleviation, treatment and diagnosis of autoimmune diseases, especially arthritic conditions.

2. Polypeptide having the following amino acid sequence:

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171          181          191          201
GVITVEESNT   FGLQLELTEG   MRFDKGYISG   YFVTDPERQE
211          221          231
AVLEDPYILL   VSSKVSTVKD   LLPLLEKVIG.
```

3. A polypeptide useful for the diagnosis of , or as immunogen against autoimmune diseases, which polypeptide is composed of 4 to 70 amino acid residues, in the amino sequence of which at least 4 of the amino acid residues are in the same relative position as the same amino acid residues are in the polypeptide of claim 2.

4. The polypeptide of claim 3, further characterized in that it comprises in its amino acid sequence at least one of amino acid residues F, D, K and G corresponding to positions 193, 194, 195 and 196 of the polypeptide of claim 2.

5. The polypeptide of claim 4 comprising in its molecule the amino acid sequence 193-234 of the polypeptide of claim 2.

6. The polypeptide of claim 4 comprising in its molecule the amino acid sequence 193-208 of the polypeptide of claim 2.

7. The polypeptide of claim 4 comprising in its molecule the amino acid sequence 180-196 of the polypeptide of claim 2.

8. Compound according to any one of claims 2 to 7 coupled to at least one radical enhancing its antigenicity and immunogenicity.

European Patent Office

EUROPEAN SEARCH REPORT

Application Number

EP 87 20 1691

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
D,Y	WO-A-8 505 034 (UNIVERSITY COLLEGE LONDON AND YEDA RESEARCH AND DEVELOPMENT CO. LTD) * Claims 1,3-5,7-17; page 2, lines 15-25; page 6, line 12 - page 7, line 15 * ---	1-15	A 61 K 37/02 A 61 K 39/02 G 01 N 33/564 G 01 N 33/68 C 07 K 13/00 C 07 K 15/00 C 12 N 15/00 C 12 N 1/20
D,Y	INFECTION AND IMMUNITY, vol. 50, no. 3, December 1985, pages 800-806, American Society for Microbiology; J.E.R. THOLE et al.: "Cloning of Myocobacterium bovis BCG DNA and expression of antigens in Escherichia coli" * Whole document * ---	1-15	
A	BIOLOGICAL ABSTRACTS, vol. 82, no. 2, 1986, page AB-444, abstract no. 13678, Biological Abstracts, Inc., Philadelphia, PA., US; F. EMMRICH et al.: "A recombinant 64 kilodalton protein of Mycobacterium bovis BCG specifically stimulates human T4 clones reactive to mycobacterial antigens", & J. EXP. MED. 163(4), 1024-1029, 1986 * Abstract * ---	1	
A	THE LANCET, vol. 2, no. 8502, 9th August 1986, pages 310-313, London, GB; T.H.M. OTTENHOFF et al.: "Evidence for an HLA-DR4-associated immune-response gene for mycobacterium tuberculosis" * Page 310, summary; page 312, lines 23-27 * --- -/-	1	

TECHNICAL FIELDS SEARCHED (Int. Cl.4)

C 12 N 7/00
C 12 N 1/20
C 12 R 1/42

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	14-12-1987	RYCKEBOSCH A.O.A.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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